Mechanistic Study of Utilization of Water-Insoluble *Saccharomyces cerevisiae* Glucans by *Bifidobacterium breve* Strain JCM1192

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ABSTRACT Bifidobacteria exert beneficial effects on hosts and are extensively used as probiotics. However, due to the genetic inaccessibility of these bacteria, little is known about their mechanisms of carbohydrate utilization and regulation. *Bifidobacterium breve* strain JCM1192 can grow on water-insoluble yeast (*Saccharomyces cerevisiae*) cell wall glucans (YCWG), which were recently considered as potential prebiotics. According to the results of ¹H nuclear magnetic resonance (NMR) spectrometry, the YCWG were composed of highly branched (1→3,1→6)-β-glucans and (1→4,1→6)-α-glucans. Although the YCWG were composed of 78.3% β-glucans and 21.7% α-glucans, only α-glucans were consumed by the *B. breve* strain. The ABC transporter (*malEFG1*) and pullulanase (*aapA*) genes were transcriptionally upregulated in the metabolism of insoluble yeast glucans, suggesting their potential involvement in the process. A nonsense mutation identified in the gene encoding an ABC transporter ATP-binding protein (MalK) led to growth failure of an ethyl methanesulfonate-generated mutant with yeast glucans. Coculture of the wild-type strain and the mutant showed that this protein was responsible for the import of yeast glucans or their breakdown products, rather than the export of α-glucan-catabolizing enzymes. Further characterization of the carbohydrate utilization of the mutant and three of its revertants indicated that this mutation was pleiotropic: the mutant could not grow with maltose, glycogen, dextrin, raffinose, cellobiose, melibiose, or turanose. We propose that insoluble yeast α-glucans are hydrolyzed by extracellular pullulanase into maltose and/or maltooligosaccharides, which are then transported into the cell by the ABC transport system composed of MalEFG1 and MalK. The mechanism elucidated here will facilitate the development of *B. breve* and water-insoluble yeast glucans as novel synbiotics.

IMPORTANCE In general, *Bifidobacterium* strains are genetically intractable. Coupling classic forward genetics with next-generation sequencing, here we identified an ABC transporter ATP-binding protein (MalK) responsible for the import of insoluble yeast glucan breakdown products by *B. breve* JCM1192. We demonstrated the pleiotropic effects of the ABC transporter ATP-binding protein in maltose/maltooligosaccharide, raffinose, cellobiose, melibiose, and turanose transport. With the addition of transcriptional analysis, we propose that insoluble yeast glucans are broken down by extracellular pullulanase into maltose and/or maltooligosaccharides, which are then transported into the cell by the ABC transport system composed of MalEFG1 and MalK. The mechanism elucidated here will facilitate the development of *B. breve* and water-insoluble yeast glucans as novel synbiotics.

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Bifidobacteria are human gut commensals and have been shown clinically to have beneficial effects, including the prevention of diarrhea (1), the alleviation of irritable bowel syndrome (2) and bowel inflammation (3, 4), and immunomodulation (5). Because of the variety of beneficial effects, bifidobacteria are now some of the most commonly used probiotics in commercial food products. Despite the wide interest, however, mechanistic studies of bifidobacteria are scarce, due to the genetic inaccessibility of most strains. The main reasons for this genetic inaccessibility include the intrinsic restriction modification systems (6–8), the thick and complex cell wall that acts as a physical barrier, and the exopolysaccharides secreted from the bacteria (9, 10).

To promote probiotic effects, bifidobacteria can be administered with prebiotics that can selectively promote their growth. The manufacture of yeast (*Saccharomyces cerevisiae*) extracts from the food industry produces large amounts of yeast cell wall remains as by-products. Nondigestible β -(1 $\rightarrow$ 3,1 $\rightarrow$ 6)- and α -glucans, including those coisolated from the yeast cell wall, were recently considered as potential prebiotics (11, 12). In our laboratory, we showed that *Bifidobacterium breve* JCM1192 was able to use insoluble yeast glucans as the sole carbon source (H. Y. Keung and T. K. Li, unpublished observation), although the underlying utilization mechanism is yet to be elucidated. It was shown that, among strains of fecal isolates, bifidobacteria were some of the most abundant microbes isolated on agar screening plates containing soluble high-amylose or high-amylopectin starch (13, 14). However, the utilization of water-insoluble α -glucans is strain specific. Prediction of carbohydrate-active enzymes by using the HMMER *hmmscan* tool (15) with the CAZy database indicated that *B. breve* JCM1192 possesses four α -glucan-debranching enzymes, including an amylo- α -1,6-glucosidase, two glycogen-debranching proteins, and an extracellularly secreted pul-lulanase. For β -glucan digestion, three β -glucosidases belonging to the glycosyl hydrolase 3 (GH3) family and a hypothetical protein belonging to GH5 were predicted. However, *B. breve* JCM1192 does not possess β -glucanases that can hydrolyze long-chain or branching β -(1 $\rightarrow$ 3,1 $\rightarrow$ 6)-glucans. A previous study showed that *B. breve* UCC2003 could utilize only cellodextrins with degrees of polymerization ranging from 2 to 5 and the capability was mainly limited by the corresponding ABC transport system (16).

While the gold standard for understanding the role of a specific gene is analysis of its mutants, successful cases of site-directed mutagenesis of bifidobacteria are still scarce and strain specific (17). Tools such as suicide vectors, plasmid artificial modification, and temperature-sensitive plasmids had been developed (18–20). However, these approaches were reported to be unsuccessful for other research groups and other bifidobacterial strains (17). Development of a site-directed mutagenesis method for a new strain is time-consuming, and the success rate is usually low. For random mutagenesis, a Tn5 transposon mutant library was reported in 2013 (21). However, transformation of strain *B. breve* JCM1192 using the same tetracycline-resistant Tn5 transposon system (with pAM5 vector provided by D. van Sinderen, University of Cork) was unsuccessful.

Recent decreases in the cost of next-generation sequencing have enabled more-comprehensive discoveries of mutations in a mutant genome. Chemical mutagenesis provides a more convenient way to generate mutants, as long as there is a practical screening system. Here, commercial yeast cell wall glucans (YCWG) were used to develop screening plates for mutant isolation. Limited amounts of glucose were added, to allow mutants with utilization deficiency to develop into visible colonies. Ethyl methanesulfonate (EMS) was used to induce mutagenesis. EMS has been used extensively to induce mutagenesis in various organisms, including bacteria, *Drosophila*, plants, and mammals (22–27). It is an alkylating agent that donates an alkyl group to guanine and causes mostly G/C to A/T transitions, resulting in adenine- and uracil-rich

FIG 1 Characterization of glycogen (oyster source) (A), YCWG (B), and *B. breve* wild-type spent YCWG mRCM (C) by ^1H NMR spectrometry. Partial spectra are shown at 4.1 to 5.5 ppm.

stop codons and hence nonsense mutations. The mutation frequency can be estimated by determining the frequency of rifampin-resistant (Rif^r) mutants. This allows the EMS concentration to be controlled for the induction of low-frequency mutations in the bacterial genome. In this study, we aimed to reveal the mechanism for the utilization of insoluble yeast cell wall glucans by *Bifidobacterium breve* JCM1192, as well as to provide insights into the feasibility of mutant genome resequencing for other mechanistic studies. The utilization mechanism revealed here will facilitate the development of potential synbiotics from water-insoluble yeast cell wall glucans and *B. breve*, making strain improvement possible.

RESULTS AND DISCUSSION

Yeast glucan utilization in *B. breve*. The structure and degree of branching (DB) of YCWG were determined by ^1H nuclear magnetic resonance (NMR) spectrometry. Interpretation of YCWG ^1H spectra was based on the chemical shifts for (1 $\rightarrow$ 4)- α -glucan, (1 $\rightarrow$ 6)- α -glucan, and yeast (1 $\rightarrow$ 3,1 $\rightarrow$ 6)- β -glucan from references 29, 34, and 66. ^1H NMR spectrometry of ultrapure glycogen (from oysters) in dimethyl sulfoxide (DMSO)- d_6 - D_2O (6:1 [vol/vol]) mixed solvent was also performed, to provide a reference chemical shift for identification of α -glucans in YCWG. The Glcp units in glycogen are linked by (1 $\rightarrow$ 4)- α -glycosidic bonds linearly, with (1 $\rightarrow$ 6)- α branching (28). Two major peaks were identified in the glycogen (Fig. 1A). The doublet at 5.136 and 5.092 ppm was assigned to H-1 of the (1 $\rightarrow$ 4)- α -linked backbone, and a single peak at 4.789 ppm was assigned to (1 $\rightarrow$ 6)- α -branching units (29). The spectrum of glycogen exhibited line broadening, which is common for high-molecular-weight polysaccharides, due to the decrease in the motional rate of larger molecules in solution (30). The DB calculated using the integration of the two peaks was 0.081, close to a previously reported result

FIG 2 Glucan contents after 7 days of prolonged incubation of *B. breve* JCM1192 (wild-type [WT]) and an uninoculated control in YCWG mRCM. Results are mean values obtained from three independent experiments, with the error bars representing standard deviations. *, significant difference (Student's *t* test, $P < 0.01$).

(31). The previously described (1→4,1→6)- α -glucan in YCWG (32) was confirmed, as indicated by the doublet at 5.134 and 5.094 ppm (Fig. 1B) and a single peak at 4.785 ppm. The DB of (1→4,1→6)- α -glucan in YCWG was 0.121, which is higher than that of glycogen in this study. The result showed that the α -glucans of YCWG were highly branched. On average, branching occurred at every eighth residue. The degree of polymerization of YCWG could not be calculated, since terminal proton resonances were not observed. This is common to high-molecular-weight samples, as the number of terminal residues is low. Free anomeric protons were reported to be undetectable in the spectrum of dextran, with a mass of 70.8 kDa (33). A majority of the (1→4)- α -glucan fraction extracted from YCWG was reported to have a mass of >100 kDa (34).

Chemical shifts of YCWG at 4.556 and 4.306 ppm were attributed to the backbone (1→3)- β -linked and branch point (1→6)- β -linked Glcp residues, respectively (66). The DB calculated from the integration of the doublet was 0.224, which is higher than reported previously. The nonreducing terminus (NRT) H-1 signal was identified as a doublet at 4.453 ppm. The ^1H NMR spectrum of the wild-type spent YCWG modified reinforced clostridial medium (mRCM) showed that, after 7 days of incubation, the α -glucan contents were exhausted, as indicated by the absence of peaks in the corresponding region. The region of β -glucan contents (4.3 to 4.6 ppm) in the spent YCWG mRCM spectrum remained unchanged (Fig. 1C), showing that *B. breve* did not utilize the β -glucan contents in YCWG.

Of the total glucan proportion of 74.3% detected in raw YCWG, 78.3% represented β -glucans and 21.7% α -glucans. After 7 days of prolonged incubation of *B. breve* with ethanol-washed YCWG in mRCM, all α -glucans (2% [wt/wt]) in the medium were completely utilized, while the β -glucan contents remained unchanged (Fig. 2). Although *B. breve* JCM1192 could utilize water-soluble gentiobiose and cellobiose (see Table S3 in the supplemental material), the results suggested that it could not utilize water-insoluble yeast cell wall β -glucans (Fig. 2). The α - and β -glucans in YCWG are highly branched and are not readily transported into bacterial cells. Therefore, utilization of these glucans requires breakdown into simpler sugars by extracellular catabolic enzymes before entry into the cells. Pullulanase can digest α -(1→4)- and α -(1→6)-glycosidic bonds extracellularly (35). However, although β -glucosidases were putatively identified in the bacterial genome, none was a debranching enzyme or was predicted to be secreted extracellularly. This might explain the difference in the utilization of α - and β -glucans by the *B. breve* strain.

Halo zones that developed around *B. breve* colonies on YCWG modified reinforced clostridial agar (mRCA) after 5 days of incubation could reach 10 mm in diameter, suggesting that the utilization of yeast α -glucans involved digestion by extracellularly secreted enzymes. Among the carbohydrate-active enzymes predicted using auto-

FIG 3 Gene map of *malR3* and proximal genes.

mated carbohydrate-active enzyme annotation (dbCAN), only two glycoside hydrolases, annotated as glycosyl hydrolase family 25 (GH25) and pullulanase, possess signal peptides, as predicted by SignalP. BLASTP results showed that the GH25 enzyme shared 76% identity with a lysozyme, while the pullulanase shared 99% identity with amylopullulanase (AapA) from *B. breve* UCC2003. This amylopullulanase consists of independently active α -amylase and pullulanase domains. In a mutant analysis (35), the α -amylase domain was shown to hydrolyze α -(1 $\rightarrow$ 4)-glucosidic linkages in starch, glycogen, and amylopectin to maltooligosaccharides, while the pullulanase domain hydrolyzed α -(1 $\rightarrow$ 6)-glucosidic linkages in pullulan to maltotriose and polymers of maltotriose.

Transcriptional analysis of insoluble yeast α -glucan utilization genes. In a comparative genomics study of transcriptional regulation of carbohydrate utilization in 10 bifidobacterial strains, a LacI transcription factor (TF) named MalR3 and two other TFs (MalR1 and MalR2) were predicted to cross-regulate maltose/maltodextrin ABC transporters, α -glucoside hydrolases such as amylomaltases and pullulanase, glycogen phosphorylase, and enzymes involved in the bifid shunt glycolytic pathway (36). Genes adjacent to *malR3* in *B. breve* JCM1192 include upstream 4- α -glucanotransferase (*malQ*), α -amylase (*agl3*), and four ABC transporter components and downstream pullulanase (*aapA*), *dnaK*, and *grpE* (Fig. 3). Reverse transcription (RT)-quantitative PCR (qPCR) results showed that the solute-binding protein gene *malE1*, transporter permease genes (*malF1*, *malG1*, and *traX*), and *aapA* were all differentially expressed (cutoff value of 4-fold induction; $P < 0.001$, Student's *t* test) in the wild-type strain grown on YCWG mRCA, compared to that grown on glucose mRCA (Fig. 4), indicating that these genes were involved in the utilization of insoluble yeast α -glucans. The greatest induction (~ 80 -fold) was observed for *aapA*.

Genome resequencing of mutants. The size of the *B. breve* JCM1192 reference genome is 2.33 Mbp, with a GC content of 58.5% (1.36×10^6 C-G base pairs). To deduce the optimal concentration of EMS to be used, the death rate and mutation frequency of *B. breve* at different EMS concentrations were recorded (Fig. S1). Three types of missense mutations were found to be responsible for the spontaneous rifampin resistance of this strain (37). Therefore, it was estimated that a Rif^r frequency of $(1/1.36 \times 10^6) \times 3 = 2.21 \times 10^{-6}$ would result in a single C-G base pair mutation per genome. The Rif^r frequency was directly proportional to the concentration of EMS used up to 1 μ l/ml [$R^2 = 0.9996$, $y = (6.686E-06)x + 8.612E-08$] (Fig. S1B). To induce one or two point mutations per genome, an EMS concentration of 0.5 μ l/ml (within the calculated range of 0.318 to 0.648 μ l/ml) was used for subsequent independent mutagenesis experiments. Independent EMS mutagenesis experiments were conducted, and approximately 80,000 colonies were screened on glucose YCWG mRCA. Mutants that could not utilize yeast glucans developed small colonies (diameters of <1 mm), in contrast to the wild-type strain (≥ 2 mm). The average death rate was $\sim 30\%$ and the average mutation frequency

FIG 4 Relative *malR3* and proximal gene transcription levels of the wild-type strain grown in glucose and YCWG mRCM. Standard deviations, calculated from three independent experiments, are indicated for each bar. *, significant difference in fold changes with the two media (Student's *t* test, *P* < 0.001).

was 3.16×10^{-6} , close to our target level. In addition to the wild-type strain, mutant M2 was selected for genome resequencing, because it could not grow with insoluble yeast glucans as the sole carbon source.

The average read coverage of our resequenced genomes was $>15\times$, which is high enough for confident identification of single nucleotide variants (SNVs) (38). Since EMS typically induces only point mutations (in particular, transition mutations), only SNVs were taken into consideration here, and insertion/deletion (indel) calling was not performed in our bioinformatics procedures. Seven nonsynonymous SNVs were identified in the wild-type strain (Table S4), five of which were also identified in the mutants. These SNVs were regarded as the parental type and were not studied further in subsequent analyses.

Mutant M2 and its revertants. Mutant M2 could not grow on medium with yeast glucans as the sole carbon source. It possessed a nonsense (ochre) mutation in the gene of a sugar ABC transporter ATP-binding protein (MalK). The C-to-T mutation resulted in a change of the amino acid glutamine to a stop codon (Fig. 5). In the

FIG 5 Confirmation and identification of the *malK* genotype in wild-type (WT), mutant (M2), and revertant (R6, R7, and R9) strains by Sanger sequencing. The codon with the mutation is indicated with a black rectangle. Also shown are the colonies on mutant screening plates after 5 days of incubation.

FIG 6 Cross-feeding experiment with the wild-type (WT) and M2 strains with yeast cell wall glucans. Strains were enumerated on glucose YCWG mRCA at different times. Results are mean values obtained from three independent experiments.

375-amino-acid-long protein sequence, the truncation occurred at residue 334, near the C terminus.

Three main types of ABC transporter systems, i.e., exporters, importers, and units involved in translation for mRNA and DNA repair, are present in bacteria (34). All ABC systems share a highly conserved nucleotide-binding domain that hydrolyzes ATP to provide energy for biological processes. ABC exporter systems are involved in the secretion of molecules, including enzymes, while ABC importers play an important role in nutrient uptake in bacteria. In bifidobacteria, ABC transporters are known to import oligosaccharides, disaccharides, and monosaccharides (18, 39–41). The failure of M2 growth with α -glucans indicated that the MalK-associated ABC transporter is responsible for either the import of α -glucans or their breakdown products into the cell or the secretion of α -glucan-catabolizing enzymes into the medium. Coculture experiments were carried out to examine the nature of the transporter. If the ABC transporter system is an exporter of catabolic enzymes, then M2 would benefit from maltose and/or maltooligosaccharides produced from the extracellular digestion of yeast glucans by the wild-type strain. In contrast, if the ABC system is an importer, then M2 would eventually be outgrown by the wild-type strain, as no carbon source would be available. After 24 h of incubation, the population size of the wild-type strain in the yeast glucan coculture had reached a maximum level (Fig. 6), while that of M2 remained relatively unchanged. M2 was outgrown by the wild-type strain after 48 h of incubation, suggesting that M2 was unable to propagate in the coculture with the wild-type strain. Therefore, it can be concluded that the MalK-associated ABC transporter was not responsible for the export of catabolic enzymes. Instead, it is speculated that this transporter took part in the internalization of α -glucans or their breakdown products.

The API CH50 panel was used to assess the carbohydrate utilization phenotype of M2. Results showed that the mutant could not ferment amygdalin, maltose, D-raffinose, starch, glycogen, or D-turanose (Table S3). In the case of cellobiose and melibiose, fermentation seemed incomplete and was delayed, compared to that of the wild-type strain. This pleiotropic phenomenon suggested that either *malK* can partner with various ABC transporter components or the ABC transporter of its constituent can import various substrates. However, as the substrates are diverse in the types of monomer, bonding (α - or β -glycosidic bonds), and degree of polymerization (disaccharides, trisaccharides, and probably oligosaccharides from the breakdown of starch and glycogen), the latter scenario is less possible. Indeed, the interactions of ATPase subunits with different membrane permeases to form individual ABC transporters were observed with Gram-positive bacteria (42–44).

To understand whether the carbohydrate utilization defect in M2 is solely due to the nonsense mutation in *malK*, we performed a complementation study as well as

FIG 7 α -Glucan contents remaining after 4 days of incubation of wild-type, M2, and revertant (R6, R7, and R9) strains in YCWG mRCM. Results are the means of three independent experiments. Different letters indicate significant differences between groups (one-way ANOVA and Tukey's test, $P < 0.01$).

isolation of revertants that regained the ability to use glucans as a carbon source. In the complementation test, a conjugation system using *Escherichia coli* SM10 λ pir (45) with the RP4 plasmid (46) was used to transfer the plasmid with the wild-type *malk* sequence into M2, according to the methodology described previously (47). However, we failed to produce a successful clone under the conditions tested. It seemed that the *B. breve* conjugant showed only transient expression of antibiotic resistance, and the plasmid could not propagate. However, three revertants (R6, R7, and R9) of M2 with different patterns of codon reversion were isolated (Fig. 5). R6 was a true revertant, with the codon CAG (and hence the glutamine residue) restored. In contrast, R7 and R9 were pseudorevertants. R7 reverted from TAG (inherited from M2) to the tryptophan-encoding TGG, while R9 reverted to the tyrosine-encoding TAT. The genotypes of the revertants were determined by Sanger sequencing of the regions flanking the SNVs of M2 and genome resequencing. The Sanger sequencing results confirmed that the revertants shared all of the SNVs found in M2, apart from the reversion of *malk*. On the genome level, new independent SNVs were identified in the revertants (Table S4). These SNVs were randomized among the revertants, and the genes involved showed no relationship to carbohydrate utilization. Therefore, the mutation and reversions of *malk* were responsible for the carbohydrate utilization phenotypes.

All revertants regained the wild-type phenotype in utilizing insoluble yeast α -glucans (Fig. 7) and API CH50 substrates (Table S3). To study whether different types of amino acid reversion affected the function of MalK, the growth of the bacterial strains with maltose, glycogen, dextrin, and raffinose was monitored. Overall, no significant differences between the wild-type strain and the revertants in growth rates (maximum slopes) or final optical density at 600 nm (OD_{600}) values ($P > 0.01$, Student's *t* test) were observed, while the growth of M2 with these carbohydrates was arrested (Fig. 8A to D). The growth rates of the strains with cellobiose, melibiose, and turanose were relatively low; therefore, the final OD_{600} values after 24 h of incubation were compared. The results showed no significant differences between the growth of the revertants with cellobiose, melibiose, and turanose and that of the wild-type strain, while the growth of M2 with those carbohydrates was significantly lower (Fig. 9). Although a second missense mutation (asparagine-335 to threonine) was found in R6 (Fig. 5), the aforementioned results showed that it did not affect the function of MalK.

Together, these results indicate that the nonsense mutation in *malk* is responsible for the defects in the utilization of cellobiose, melibiose, raffinose, turanose, maltose, and the breakdown products of glycogen and dextrin, presumably due to impairment in transport of these carbohydrates into the cell through the MalK-associated ABC transporter system. The effects of reversions with the three amino acid types were similar, and the revertants showed no differences in the utilization of the tested

A) Maltose

B) Glycogen

C) Dextrin

D) Raffinose

FIG 8 Growth curves for wild-type (WT), M2, and revertant (R6, R7, and R9) strains on 1% maltose (A), glycogen (B), dextrin (C), and raffinose (D) mRCM. Results are the means of three independent experiments.

carbohydrates, compared to the wild-type strain, suggesting that the replacement of glutamine with tryptophan or tyrosine at this position was not crucial for the general function of the protein.

Among the carbohydrates tested, raffinose and melibiose contain α -(1 $\rightarrow$ 6)-linked galactose moieties. In a transcriptomic study of *B. breve* UCC2003, a raffinose ABC transport system, namely, *rafBCD*, was found to be upregulated in the presence of either raffinose or melibiose (48). In accord with our experimental results, RafBCD and MalK may constitute an ABC transport system responsible for the import of raffinose and melibiose. Similarly, MalK may form an ABC transporter system with ClcEFG for the

FIG 9 Final OD₆₀₀ values following 24 h of growth of various strains with 1% cellobiose, melibiose, and turanose. Results are the means of three independent experiments, with error bars showing the standard deviations. WT, wild-type. *, significant difference (Student's *t* test, *P* < 0.001).

FIG 10 Proposed pathway for the catabolism and regulation of the utilization of water-insoluble yeast α -glucans by *B. breve* JCM1192. α -Glucans are digested extracellularly by pullulanase (AapA) into maltose and/or malto-oligosaccharides (MOS), which are then transported into the bacterial cell by an ABC transporter system composed of MalEFG1 and MalK. Green arrows indicate the pathways studied in this work. Red double strokes indicate the affected translation of MalK.

import of cellodextrin (16). Further studies with a glutathione S-transferase (GST) pulldown assay will identify proteins that interact with MalK.

The *malK* gene did not colocalize with any ABC transporter gene cluster, and it showed constitutive expression across glucose- and α -glucan-based media, at intermediate levels (Fig. 4). In several bifidobacterial carbohydrate utilization studies, the transcription of ABC transporter solute-binding proteins and permeases was found to be regulated by TFs; however, no ATPase was coregulated (16, 41, 48). Since *malK* was found to be responsible for the transport of multiple sugars in our experiment, its constitutive expression may provide an advantage to the bacteria in acquiring various scarce resources quickly inside the intestine (49).

Conclusions. We propose that, in *B. breve*, water-insoluble α -glucans are catabolized extracellularly by pullulanase into maltose and/or maltooligosaccharides, which are then transported into the bacteria through an ABC transporter system (Fig. 10). Constitutively expressed MalK is crucial for the transport of cellobiose, melibiose, turanose, and raffinose, in addition to maltose and maltooligosaccharides. Chemical mutagenesis coupled with next-generation sequencing can act as an alternative to targeted mutagenesis in generating mutants for definitive mechanistic studies. Our work will facilitate the development of potential synbiotics from water-insoluble yeast glucans and *B. breve*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Bifidobacterium breve* strain JCM 1192 was purchased from the RIKEN BioResource Center (Ibaraki, Japan) and stored in reinforced clostridial medium (RCM) (Oxoid) with 30% glycerol at -80°C until use. The bacteria were subcultured twice in RCM before experiments. Bifidobacterial cultures were incubated at 37°C under anaerobic conditions maintained with an Anaero-Gen 2.5-liter system (Oxoid), in anaerobic jars (agar) or 1:50 Oxyrase (Oxoid) (liquid culture).

EMS mutagenesis. (i) Determination of EMS concentrations for mutagenesis. Overnight cultures of *B. breve* with an OD_{600} of ~ 1 were washed twice with phosphate-buffered saline (PBS). EMS (product number M0880; Sigma) solution was then added at a concentration of 0 (control), 0.5, 1, 2, 4, 7, 10, 15, or 20 $\mu\text{l}/\text{ml}$, and the cultures were incubated for 1.5 h at 37°C , with shaking at 80 rpm. The bacteria were then again washed twice with PBS. To determine the death rate with EMS, the bacteria, after appropriate dilution, were spread on reinforced clostridial agar (RCA) and incubated anaerobically at 37°C for 24 h. To determine the mutation frequency, the bacteria were recovered in RCM for 16 h with Oxyrase at 37°C , spread on rifampin (50 $\mu\text{g}/\text{ml}$) RCA, and incubated anaerobically at 37°C for 24 h. The mutation rate was inferred by determining the frequency of Rif^r mutants.

(ii) Mutant screening plates. To allow growth and differentiation of mutant colonies, glucose was added to the mutant screening plates. Commercial baker's YCWG were purchased from Angel Yeast

(Hubei, China). Water-soluble YCWG and glucose were removed as follows: 3 g of YCWG was hydrated with 100 ml of 14% ethanol for 30 min, with a magnetic stirrer. The mixture was diluted to 500 ml with deionized water and heated to 80°C for 3 h. The mixture was then centrifuged at $10,000 \times g$ for 10 min to remove the supernatant. Deionized water was added to the bottom mixture to make up a total volume of 200 ml. The mixture was then mixed well for autoclaving. Modified RCA (mRCA) and mRCM were used as the basal growth media, in which carbohydrate sources were excluded based on the formula designed by Hirsch and Grinstead (50). The autoclaved yeast glucan mixture was mixed with separately autoclaved mRCA with 30 mg/liter Congo red and 0.2- μ m-filtered 0.01% (final concentration [wt/vol]) glucose to a final volume of 500 ml, for preparation of screening plates.

(iii) Mutagenesis and mutant screening. Independent mutagenesis experiments were carried out using the same procedures as described for EMS concentration determinations, except that no (control) or 0.5 μ l/ml EMS was used. The recovered bacteria were diluted appropriately according to the OD₆₀₀ values and were spread on the mutant screening plates. The plates were then incubated anaerobically at 37°C for 4 days. The death rate and mutation frequency were recorded for each mutation experiment.

Mutants were isolated if the sizes of their colonies or halos were different from those of the wild-type strain. The phenotypes of mutants were reconfirmed by patching a colony on new mutant screening plates. Phenotype-confirmed mutants were inoculated in RCM; overnight cultures were washed and resuspended in RCM with 30% glycerol and stored at -80°C. The growth rates of mutants in mRCM with 1% (wt/vol) glucose were analyzed to avoid mutants with defects in glucose metabolism that might involve nontarget enzymes in the bifid shunt pathway, a unique central hexose fermentation pathway in bifidobacteria (51).

Genome sequencing. (i) DNA extraction. Bead beating and mutanolysin digestion were used to obtain intact genomic DNA. Briefly, 1-ml aliquots of overnight cultures of the wild-type and mutant strains were centrifuged at $5,000 \times g$ for 10 min and then washed twice with PBS. The cell pellets were resuspended in 200 μ l TE buffer (30 mM Tris-HCl, 1 mM EDTA [pH 8]). Mutanolysin (75 U) (product number M9901; Sigma) and 4 μ l RNase A (100 mg/ml) (Qiagen) were added to each sample, and the mixtures were incubated at 37°C for 30 min. Cell suspensions were transferred to 2-ml screw-cap tubes filled with 0.1-mm-diameter glass beads (Biospec, Bartlesville, OK, USA), and cells were physically disrupted with a Bioprep-24 homogenizer (Allsheng Instruments, Hangzhou, China) for 3 cycles (20 s of shaking, with 1-min intervals on ice). Ice-cold absolute alcohol (500 μ l) was added to the cell lysates, and DNA was extracted and column purified using the DNeasy DNA extraction kit (Qiagen), following the manufacturer's instructions.

(ii) Ion Torrent genome sequencing. Genomic library preparation was performed using the Ion PGM Sequencing 400 kit (Life Technologies). Genomic DNA from the strains was fragmented by sonication. The overall fragment length was checked with an Agilent 2100 bioanalyzer, with a high-sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA). Fragments of ~400 bp were selected with E-Gel SizeSelect 2% agarose gels (Thermo Fisher). The selected libraries were amplified for eight cycles with the reagents supplied in the Ion Plus Fragment kit (Thermo Fisher) and then were purified by using Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics, Germany) to remove primer dimers. The amounts of DNA fragments per microliter were calculated from the concentration and average size of each amplicon, and libraries were created by using the Ion Plus Fragment Library kit (Life Technologies, Italy). Sample-specific barcodes were added to each sample. Emulsion PCR was carried out using the Ion OneTouch TM 400 Template kit (Life Technologies). Sequencing of the amplicon libraries was carried out with Ion 318 chip kit v2 using an Ion Torrent PGM system, with the Ion PGM Hi-Q kit (Life Technologies).

Bioinformatics. (i) Read mapping and SNV calling. Raw reads with low quality (<Q20) were removed with the FASTQ Quality Filter (FASTX-Toolkit). Trimmed high-quality reads were mapped to the *Bifidobacterium breve* JCM1192 reference genome (GenBank accession number [NZ_AP012324](#)), using the Burrows-Wheeler aligner (BWA) tools (67). The BWA default parameters for mapping were used. SNVs were identified by SAMtools (52), with stringent options to minimize false-positive results. Only variants with quality scores higher than 25 from bcftools calls were reported. Variant annotation and effect prediction were carried out using SnpEff v4.0 (53).

(ii) Prediction of carbohydrate-active enzymes and signal peptides. The amino acid sequences of *B. breve* were annotated using dbCAN (54) with the CAZy database (55). Domain prediction was carried out with HMMER v3.1b1 (56). Prediction of the presence and location of signal peptide cleavage sites in the amino acid sequence was performed using SignalP 4.1 (57).

SNV confirmation by Sanger sequencing. Primers were designed to amplify the regions flanking SNVs of interest (Table S1) by PCR, using genomic DNA from the mutants. Briefly, a 50- μ l reaction mixture contained 1 μ l of 10 μ M forward and reverse primers, 10 μ l 5 $\times$ PrimeSTAR GXL buffers (TaKaRa Bio Inc., Otsu, Shiga, Japan), 4 μ l 2.5 mM deoxynucleoside triphosphate (dNTP) mixture, 0.1 μ l genomic DNA, and 2.5 U PrimeSTAR GXL polymerase (TaKaRa Bio Inc.). The PCR conditions were as follows: initial denaturation at 98°C for 5 min, 30 cycles of denaturation at 98°C for 1 min, annealing at 55°C for 15 s, and extension at 68°C for 50 s, and final extension at 68°C for 10 min. The quality and size of the PCR products were checked by 1.5% (wt/vol) agarose gel electrophoresis. Bands with expected sizes were excised and gel purified using a gel extraction kit (Geneaid Biotech Ltd., Taipei, Taiwan). Purified PCR products were sequenced by Sanger sequencing (Beijing Genomic Institute, Hong Kong) for SNV verification.

Characterization of insoluble yeast cell wall glucans. DMSO-*d*₆ (99.9 atom % D) and D₂O (99.8 atom % D) were purchased from Acros Organics (Geel, Belgium). Glycogen, YCWG, and wild-type spent YCWG were prepared by dissolving 30 mg of the respective material in 1 ml of DMSO-*d*₆-D₂O (6:1) at 60°C for 30 min, in 5-mm-diameter glass sample tubes (500 MHz; Wilmad). ¹H NMR spectra were recorded

at 400.13 MHz in a Bruker AV400 NMR spectrometer operating at 80°C. The chemical shifts were referenced internally to DMSO- d_6 at 2.49 ppm for ^1H .

For characterization of *B. breve* utilization, overnight cultures of the wild-type strain were inoculated at 2% (vol/vol) in 10 ml YCWG (1.2% [wt/vol]) mRCM with 200 μl Oxyrase, in Hungate anaerobic culture tubes, and incubated for 5 days at 37°C, with shaking at 70 rpm. At the end of the incubation, the cultures were collected by centrifugation at $3,000 \times g$ and then washed twice with 10 ml sterile double-distilled water. The cultures were freeze-dried for NMR analysis.

The DB can be calculated from the peak integrals of the assigned H-1 peaks by using the following equation: DB = peak area for H-1 protons of terminal residues in the side chain/peak area for all H-1 protons in the backbone.

Determination of α - and β -glucan utilization. Glucan growth medium was prepared as for the mutant screening plates, except that no agar or glucose was added. Overnight cultures of wild-type and mutant strains were inoculated at 2% (vol/vol) in 10 ml YCWG (0.6% [wt/vol]) mRCM with Oxyrase, in Hungate anaerobic culture tubes, and incubated for 4 or 7 days, with shaking at 70 rpm. At the end of the incubation, the cultures were freeze-dried and 100-mg aliquots of well-mixed samples were weighed accurately for assays. The Megazyme β -glucan (yeast and mushroom) assay kit was used to quantify (1 $\rightarrow$ 3,1 $\rightarrow$ 6)- β -glucans and α -glucans in YCWG and their utilization, according to the manufacturer's instructions. The amount of β -glucans was determined as the difference between the amount of total glucans and the amount of α -glucans. Briefly, total glucans (1 $\rightarrow$ 3,1 $\rightarrow$ 6)- β -D-glucans, (1 $\rightarrow$ 3)- β -D-glucans, α -glucans, sucrose, and glucose) were solubilized in concentrated (37% [10 N]) hydrochloric acid and then extensively hydrolyzed with 1.3 N HCl at 100°C for 2 h. After dilution and centrifugation, an aliquot was removed for incubation with a mixture of highly purified exo-1,3- β -glucanase and β -glucosidase, to complete the hydrolysis to D-glucose. Glucose contents were then determined colorimetrically by incubating the samples with glucose oxidase plus peroxidase and 4-aminoantipyrine (GOPOD) at 40°C for 20 min and measuring the absorbance at 510 nm. α -Glucan, sucrose, and free glucose levels were determined as follows: the samples were solubilized in 2 M potassium hydroxide for 20 min in an ice water bath, followed by amyloglucosidase and invertase incubation at 40°C for 30 min to release glucose. Glucose contents were then determined colorimetrically by incubating the samples with GOPOD at 40°C for 20 min and measuring the absorbance at 510 nm. The assays were carried out in triplicates for each strain.

Carbohydrate utilization profiling. The ability of the wild-type and mutant strains to utilize various carbohydrates was characterized using an API CH50 system (bioMérieux, France). Two microliters of overnight cultures of the strains was washed twice and resuspended in 500 μl PBS for OD measurements. An appropriate volume of inoculation was added to the CHL medium, according to the manufacturer's instructions, and the samples were mixed well with a vortex mixer. All ampules were filled with approximately 135 μl of suspension and incubated at 37°C for 48 h. Results were recorded at 24 and 48 h. Since fermentation-led color changes were gradual, from dark blue to yellow at the endpoint, intermediate changes (various intensities of green color) were marked + and the endpoint was marked ++. Tests were carried out in duplicate for each strain.

Glucose (product number G8270), maltose monohydrate (product number M5885), dextrin (product number D2256), raffinose pentahydrate (product number R0250), cellobiose (product number 22150), melibiose (product number 63630), and turanose (product number T2754) were purchased from Sigma. Glycogen from an oyster source (part number 16445) was purchased from Affymetrix (Tokyo, Japan). All carbohydrates were filtered through a 0.22- μm filter (Millipore, Germany). Modified RCM and RCA (50) were used as the base media for carbohydrate utilization studies.

Bacterial growth profiles were determined by either kinetics (cell density over time) or final optical density values for the tested strains with different carbohydrates. Experiments for final OD₆₀₀ measurements were set up with each tube containing 1% (wt/vol) carbohydrate source, 2% Oxyrase (Oxoid), and 1% (vol/vol) overnight culture of the bacterial strain in mRCM, in a final volume of 5 ml. Cultures were incubated at 37°C for 24 h. Uninoculated mRCM samples with each carbohydrate were used as negative controls. For the kinetic study, 96-well plates were set up as in the final OD₆₀₀ study, except that the total volume of culture medium was 200 μl . The program for kinetic OD₆₀₀ measurements was set with Magellan software on a Tecan M200 Infinite Pro instrument, using 1-h intervals with 30 s of orbital shaking and incubation at 37°C. The growth rates of the strains with various carbohydrates were deduced using grofit (58), with R v3.1.1 (59).

Coculture experiments. Wild-type and mutant strains were grown anaerobically in glucose mRCM overnight at 37°C. The cell density of the cultures was adjusted to an OD₆₀₀ of 2.6 with mRCM, and 500 μl of each culture was inoculated to 50 ml YCWG (2.4% [wt/vol]) mRCM. One-milliliter aliquots of cultures were sampled at different times, diluted, and spread on glucose (0.01% [wt/vol]) YCWG (0.6% [wt/vol]) mRCA plates. The plates were incubated anaerobically at 37°C for 4 days, and the numbers of colonies for the wild-type strain (colony size of ≥ 2 mm) and mutants (≤ 1 mm) were recorded.

Transcriptional analysis. (i) RNA extraction from the wild-type strain grown on yeast glucans. Overnight cultures of the bacteria were diluted appropriately, spread on glucose mRCA and yeast glucan mRCA, and incubated anaerobically at 37°C. Colonies were removed from the agar plates, with a glass spreader previously heated at 300°C, to 1.5 ml PBS with RNeasy Protect Bacteria reagent (Qiagen). Total RNA was extracted from bacteria grown to the mid-log phase using the RNeasy Protect Bacteria minikit (Qiagen). Mutanolysin (75 U) was added before the bead beating procedure. After elution of RNA, DNA was removed using the Ambion Turbo DNA-free kit, according to the manufacturer's instructions. The concentration and purity (A_{260}/A_{280}) of RNA samples were measured with a NanoDrop spectrophotometer (Thermo Scientific), for calculation of the volume needed for reverse transcription. The quality of RNA

samples was analyzed using an Agilent 2100 bioanalyzer. The 16S/23S ratio and RNA integrity number were calculated for each sample. Only samples with RNA integrity numbers of ≥ 8 were used for subsequent experiments. RNA samples were stored immediately, in aliquots, at -80°C until use.

(ii) Reverse transcription. cDNA was synthesized with 150 ng total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), according to the supplier's instructions. The reaction mixture was incubated at 25°C for 5 min, followed by 42°C for 30 min and 85°C for 5 min for denaturation of the reverse transcriptase enzyme.

(iii) Primer design and verification. Primers for target gene amplification were designed using Primer3 v4.0.0 (60). The criteria for primer design were a melting temperature (T_m) between 58 and 63°C and an amplicon size of approximately 90 to 125 bp. Primers were checked for potential off-target amplification products using NCBI Primer-BLAST, with a user-guided database (61). The primers used for RT-qPCR are described in Table S2 in the supplemental material. Verification of primers was carried out using a 10-fold dilution of synthesized cDNA. Briefly, a 25- μl PCR mixture was set up with 5 μl of $5\times$ GoTaq buffer (Promega), 1.2 μl of 25 mM MgCl_2 , 0.6 μl of dNTPs (10 mM each), 1 μl of 10 μM forward and reverse primers, 1 μl of cDNA (10 ng/ μl), and 0.25 μl of GoTaq Flexi DNA polymerase (Promega). The PCR program was as follows: 95°C for 2 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and finally 72°C for 3 min. Three microliters of the PCR amplicons were analyzed by 1.5% agarose gel electrophoresis. Primers were eliminated when multiple bands or severe primer dimers were detected.

(iv) Real-time PCR. The experimental design, procedures, and data analyses followed the MIQE guidelines whenever applicable (62). Reaction mixtures (20 μl each) were set up in a MicroAmp optical 96-well reaction plate with barcode (Applied Biosystems), and each mixture contained 10 μl PowerUp SYBR green master mix ($2\times$; Applied Biosystems), 1 μl of the diluted cDNA, and 1 μl of 5 mM forward and reverse primers. Real-time PCRs were performed with an ABI 7500 Fast real-time PCR system (Applied Biosystems). Three independent biological replicates were assayed for each strain under each experimental condition. The qPCRs for each target gene were performed in technical duplicates, with a no-template control and a no-reverse transcriptase control. The qPCR program was as follows: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed by increasing the temperature from 60°C to 90°C . The amplification efficiency of each primer was estimated with standard curves using serial dilutions of genomic DNA template and then was calculated using the slope of a linear regression model ($10^{-1/\text{slope}}$) (63). Threshold cycle (C_T) values were used for stability comparisons of candidate reference genes using BestKeeper (64). Expression levels were normalized to the geometric mean of the 16S rRNA, *recA*, and *purC* C_T values for the wild-type strain grown in glucose medium (65).

Statistical evaluation. Results were expressed as mean values and standard deviations from independent triplicates. Student's *t* test analyses were performed using the statistical analysis package in Microsoft Excel 2010. One-way analysis of variance (ANOVA) and Tukey's test were performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL).

Accession number(s). The genome data sets have been deposited in the NCBI SRA database, with the accession number [SRP082348](https://doi.org/10.1128/AEM.03442-16).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.03442-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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